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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12Q 1/02, 1/24	A1	(11) International Publication Number: WO 96/10091 (43) International Publication Date: 4 April 1996 (04.04.96)

(21) International Application Number: PCT/US95/12316

(22) International Filing Date: 25 September 1995 (25.09.95)

(30) Priority Data:
314,760 29 September 1994 (29.09.94) US

(60) Parent Application or Grant
(63) Related by Continuation
US 314,760 (CON)
Filed on 29 September 1994 (29.09.94)

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(81) Designated States: CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: A HIGH CAPACITY SCREEN FOR IMMUNOREGULANTS

(57) Abstract

A process for obtaining reproducible membrane potential measurements for immunoregulants, which depolarize the membrane potential of human T cells by blocking potassium channel $K_v1.3$. A method for analyzing compounds for activity as immunoregulants using a high capacity screening technique.

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TITLE OF THE INVENTION

A HIGH CAPACITY SCREEN FOR IMMUNOREGULANTS

BACKGROUND OF THE INVENTION

Immunoregulatory abnormalities have been shown to exist in a wide variety of "autoimmune" and chronic inflammatory diseases, including systemic lupus erythematosis, chronic rheumatoid arthritis, type I and II diabetes mellitus, inflammatory bowel disease, biliary cirrhosis, uveitis, multiple sclerosis and other disorders such as Crohn's disease, ulcerative colitis, bullous pemphigoid, sarcoidosis, psoriasis, ichthyosis, and Graves ophthalmopathy. Although the underlying pathogenesis of each of these conditions may be quite different, they have in common the appearance of a variety of autoantibodies and self-reactive lymphocytes. Such self-reactivity may be due, in part, to a loss of the homeostatic controls under which the normal immune system operates.

Similarly, following a bone-marrow or an organ transplantation, the host lymphocytes recognize the foreign tissue antigens and begin to produce antibodies which lead to graft rejection.

One end result of an autoimmune or a rejection process is tissue destruction caused by inflammatory cells and the mediators they release. Anti-inflammatory agents such as NSAID's and corticosteroids act principally by blocking the effect or secretion of these mediators but do nothing to modify the immunologic basis of the disease. On the other hand, cytotoxic agents, such as cyclophosphamide, act in such a nonspecific fashion that both the normal and autoimmune responses are shut off. Indeed, patients treated with such nonspecific immunosuppressive agents are as likely to succumb from infection as they are from their autoimmune disease.

Cyclosporin A, which was approved by the US FDA in 1983, is currently the leading drug used to prevent rejection of transplanted organs. The drug acts by inhibiting the body's immune system from mobilizing its vast arsenal of natural protecting agents to reject the transplant's foreign protein. Though cyclosporin A is effective in fighting transplant rejection, it is nephrotoxic and is known to cause

several undesirable side effects including kidney failure, abnormal liver function and gastrointestinal discomfort.

Potassium channels modulate a number of cellular events such as muscle contraction, neuro-endocrine secretion, frequency and duration of action potentials, electrolyte homeostasis, and resting membrane potential. These channels comprise a family of proteins that have been classified according to their biophysical and pharmacological characteristics. Inhibition of K^+ channels, in their role as modulators of the plasma membrane potential in human T-lymphocytes, has been postulated to play a role in eliciting immunosuppressive responses. In regulating membrane potential, K^+ channels play a role in the regulation of intracellular Ca^{+2} homeostasis, which has been found to be important in T-cell activation. The screening for inhibitors of the human T-lymphocyte K^+ channels is underdeveloped, due to the paucity of high capacity screens.

Functional voltage-gated K^+ channels can exist as multimeric structures formed by the association of either identical or dissimilar subunits. This phenomena is thought to account for the wide diversity of K^+ channels found in different tissues. Despite the rapid advances in the molecular biology of K^+ channels, subunit compositions of native K^+ channels and the physiologic role that particular channels play are, in most cases, still unclear. To address this issue of finding compounds which selectively inhibit Kv1.3, the screen identifies compounds which have the same biological inhibition profile as MgTX. The present screen utilizes a phenonmenon which is dependent on Kv1.3 block observed with MgTX.

The $K_v1.3$ channel is a voltage-gated potassium channel that is found in neurons, blood cells, osteoclasts and T-lymphocytes. The Chandy and Cahalan laboratories proposed a hypothesis that blocking the $K_v1.3$ channel would illicit an immunosuppressant response. (Chandy et al., J. Exp. Med. 160, 369, 1984; Decoursey et al., Nature, 307, 465, 1984). However, the K^+ channel blockers employed in their studies were non-selective. Until the present invention, no high capacity fluorescent screen for the $K_v1.3$ channel exists. Although a laboratory (Price et al.,

Proc. Natl. Acad. Sci. USA, 86, 10171, 1989) showed that charybdotoxin would block $K_V1.3$ in human T cells, charybdotoxin was subsequently shown to inhibit four different K^+ channels ($K_V1.3$ and three distinct small conductance Ca^{2+} activated K^+ channels) in human T lymphocytes, limiting the use of this toxin as a probe for the physiological role of $K_V1.3$ (Leonard et al., Proc. Natl. Acad. Sci. USA. 89, 10094, 1992). Since MgTX is a selective $K_V1.3$ inhibitor, it is useful to show that blocking of $K_V1.3$ will inhibit T cell activation (Lin et al., *J. Exp. Med*, **177**, 637, 1993).

A thirty-nine amino acid peptide. Margatoxin (MgTX), has been purified to homogeneity from venom of the scorpion Centruroides margaritatus. The gene encoding MgTX is constructed, and this gene is expressed in E. coli to produce recombinant MgTX. MgTX is a potent and selective inhibitor of a voltage-dependent K^+ channel present in human lymphocytes. MgTX exhibits immunosuppressant activity with human T-lymphocytes, and is useful as an immunosuppressant, in modeling nonpeptidyl K^+ channel blockers, and in establishing biochemical assays based on ligand binding or other protocols with which to screen for other novel modulators of voltage dependent K^+ channels in lymphocytes and other tissues including the brain. As an immunosuppressant, MgTX-like compounds would be useful in the treatment of autoimmune diseases, as well as to prevent the rejection of foreign organ transplants and/or related afflictions, diseases and illnesses.

The fluorescent screen of the present invention represents a unique tool with which to identify chemical species which block the function of $K_V1.3$. This channel has been identified as the major voltage-dependent K^+ conductance in peripheral human T-lymphocytes. Human T-lymphocytes contain, in addition to $K_V1.3$, several distinct small-conductance Ca^{2+}-activated K^+ channels. ChTX also blocks these channels. Thus, ChTX-like compounds are not selective inhibitors of $K_V1.3$. MgTX has recently been demonstrated to depolarize human T cells (Leonard et al., *Proc. Natl. Acad. Sci. U. S. A.* 89, 10094, 1992) and to prevent activation and proliferation of these cells mediated by Ca^{2+}-dependent pathways (Lin et al., *J. Exp. Med.*, **177**, 637, 1993).

Compounds which biochemically behave like MgTX should be specific blockers of the $K_V1.3$ channel.

Venom of the new world scorpion <u>Centruroides margaritatus</u> was determined to contain an activity selectively directed against voltage-dependent K^+ channels: it inhibited binding of $[^{125}I]$ChTX to $K_V1.3$ channels in rat brain synaptosomal membranes, but not to Maxi-K channels in smooth muscle sarcolemma. MgTX is structurally related to other known K^+ channel blocking peptides, but is distinguished by its potent and selective blockade of $K_V1.3$. Given these properties, MgTX represents a useful tool for studying the physiologic role of $K_V1.3$.

MgTX inhibits T lymphocyte activation through calcium activated pathways (Lin C.S., R.C. Boltz, J.T. Blake, M. Nguyen, A. Talento, P.A. Fischer, M.S. Springer, N.H. Sigal, R.S. Slaughter, M.L. Garcia, G.J. Kaczorowski, and G.C Koo. 1993. Voltage-gated potassium channels regulate calcium-dependant pathways involved in human T lymphocyte activation. J Exp Med 177:637.). MgTX has also been shown to block the Kv 1.3 channel resulting in an average depolarization of the membrane potential from a resting potential of -50 mV to -30 millivolts(Leonard R.J., M.L. Garcia, R.S. Slaughter, and J.P. Reuben. 1992. Selective blockers of voltage-gated K^+ channels depolarize human T lymphocytes: mechanism of the antiproliferative effect of charybdotoxin. Proc. Natl. Acad. Sci. USA 89: 10094.). Depolarization of T lymphocytes with extra-cellular potassium has also been shown to inhibit calcium influx (Hess S.D., M. Oortgiesen, and M.D. Cahalan.1993 Calcium oscillations in human T and natural killer cells depend upon membrane potential and calcium influx. J.Immunol. 150: 2620.) and activation(Freedman B.D., M.A.Price, and C.J. Deutsch. 1992 Evidence for voltage modulation of IL-2 production in mitogen stimulated human peripheral blood lymphocytes. J. Immunol. 149: 3784; Gelfand E.W., R.K. Cheung, G.B. Mills, and S.Grinstein. 1987. Role of membrane potential in the response of human lymphocytes to phytohemagglutinin. J. Immunol. 138: 527.).

It has been postulated that the effect of MgTX on T cell activation was due to its ability to depolarize the T cell and thereby affect

the calcium transient. However, this depolarization by MgTX was thought not be sufficient to inhibit activation. It has also been shown that blocking this channel with MgTX results in an initial depolarization which is followed by a second depolarization upon T-cell receptor cross linking. The instant invention utilizes the direct correlation of MgTX induced depolarization with the inhibition of T cell activation.

Previously it has been shown that pre-incubation with MgTX results in an inhibition of the intra-cellular calcium transient in human T cells activated via cross linking of cell surface molecules (Lin C.S., R.C. Boltz, J.T. Blake, M. Nguyen, A. Talento, P.A. Fischer, M.S. Springer, N.H. Sigal, R.S. Slaughter, M.L. Garcia, G.J. Kaczorowski, and G.C Koo. 1993. Voltage-gated potassium channels regulate calcium-depermdant pathways involved in human T lymphocyte activation. J Exp Med 177:637.).

It has also been observed that within the heterogeneity of the calcium response, the MgTX reduction in the Ca^{++} transient was in the peak region of the curve. It appeared to be due to a more noticeable reduction in cells exhibiting high $[Ca^{++}]_i$ rather than an overall suppression of the whole population. It has been inferred that a sub-population exhibiting the maximal initial $[Ca^{++}]_i$ was affected by MgTX. Rabinovitch *et al.* have demonstrated that CD4 cells have a greater mean calcium response than CD8 cells(Rabinovitch P.S., C.H. June, A. Grossmann, and J.A. Ledbetter. 1986. Heterogeneity among T cells in intra-cellular free calcium after mitogen stimulation with PHA or anti-CD3. simultaneous use of indo-1 and immunofluorescence with flow cytometry. J Immunol 137: 952.). The possibility existed that the observed reduction in the mean calcium transient was a result of a preferential effect on the CD4 or other subsets.

The application of combined membrane potential using DiBAC4(3) and cell surface markers on human T lymphocytes has been demonstrated (Wilson, H.A., and T.M. Chused. 1985. Lymphocyte membrane potential and Ca2+ sensitive potassium channels described by Oxinol dye fluorescence measurements . J Cell Physiol 125: 72-81; Rabinovitch P.S., and C.H. June. 1990. "Measurement of intra-cellular

ionized calcium and membrane potential." In Flow Cytometry and Cell Sorting, Second Edition, M. Melamed, T. Lidmo, M. Mendelsohn , eds. John Wiley & Sons, Inc. New York, p. 651.).

This invention combines cell surface markers with membrane potential measurement to directly follow modulation of membrane potential by $K_V1.3$ blockers in T cell subsets. Alternatively, digital imaging microscopy, can be utilized to follow the effects of $K_V1.3$ blockers on depolarization of the single lymphocytes.

SUMMARY OF THE INVENTION

A process for screening for an immunoregulant compound that modulates T cell activation which comprises measuring the effect of the immunoregulant compound on membrane potential. The screen utilizes the newly observed phenomenon of additional depolarization of the T cell membrane potential in the presence of $K_V1.3$ blockers which accompanies T cell receptor crosslinking in susceptible T cell subset(s). The screen utilizes multiparameter fluorescence flow cytometry to follow this depolarization on only these susceptible T cell subsets. Alternatively, susceptible T cells purified by magnetic cell sorting can be followed using fluorescent membrane potential dyes in a 96-well confocal fluorescent plate reader.

BRIEF DESCRIPTION OF THE FIGURE

Figure 1. Mean Depolarization Time Course of Human T Cells effected by MgTX and by anti-CD3 Cross-Linking Induced Intracellular Calcium rise. Shown are media control, MgTX inhibited and Potassium inhibited Human T Cells.

Demonstration of an increase in the mean depolarization of whole human T cells effected by anti-CD3 crosslinking in the presence of MgTX. Figure 1 shows that there is an increase in the mean depolarization of whole human T cells with the initial exposure to MgTX (triangles arrow 1). In addition, there is a further depolarization effected by anti-CD3 crosslinking (arrow 2) in the presence of MgTX. This depolarization is to the level achieved when cells are exposed to 80mM

K+ (solid circles). No depolarization is observed upon crosslinking control cultures (squares). This pattern is reflective of compounds which block Kv1.3. This can be utilized for screening for Kv1.3 blockers using unseparated human T cells with discriminating antibodies with flow cytometry or on separated sensitive subsets with a confocal fluorescence spectrofluorimeter.

DETAILED DESCRIPTION OF THE INVENTION

This invention relates to a process for screening for immunoregulant compounds that modulate T cell activation which comprises measuring the effect of the immunoregulant compound on membrane potential.

An embodiment of this invention is the process for screening for immunoregulant compounds that modulate T cell activation of whole T cell preparations which comprises the steps of:

(a) tagging a resistant T cell subset of the whole T cell preparation with one or two fluorescent antibody cell surface markers;

(b) staining the tagged whole T cell preparation with a fluorescent membrane potential dye about 1 to about 250 nM per 2 x 10^6 T cells per mL;

(c) loading the fluorescent-stained, tagged whole T cell preparation into an analytical instrument capable of measuring fluorescence;

(d) measuring a baseline membrane potential dye fluorescence of the fluorescent-stained, tagged whole T cell preparation;

(e) adding the immunoregulant compound to the fluorescent-stained, tagged whole T cell preparation;

(f) measuring the immunoregulant compound induced membrane potential dye fluorescence of the fluorescent-stained, tagged whole T cell preparation containing the immunoregulant compound after about 30 seconds to about 5 minutes;

(g) adding anti-CD3 antibody or a biotinylated anti-CD3 to the fluorescent-stained, tagged whole T cell preparation containing the immunoregulant compound;

(h) incubating the anti-CD3 antibody or a biotinylated antiCD-3, fluorescent-stained, tagged whole T cell preparation containing the immunoregulant compound for about 3 to about 10 minutes;

(i) adding a crosslinker antibody or avidin to the anti-CD3 antibody or a biotinylated anti-CD3 labeled, fluorescent-stained, tagged whole T cell preparation containing the immunoregulant compound;

(j) incubating the crosslinker antibody or avidin the anti-CD3 antibody or a biotinylated anti-CD3 labeled, fluorescent-stained, tagged whole T cell preparation containing the immunoregulant compound for about 2 minutes; and

(k) measuring the integrated anti-CD3 induced membrane potential dye fluorescence of the antibody crosslinked or avidin crosslinked anti-CD3 labeled, fluorescent-stained, tagged whole T cell preparation containing the immunoregulant compound.

A second embodiment is the process for screening for immunoregulant compounds that modulate T cell activation of T cell preparations which comprises the steps of:

(a) tagging a resistant T cell subset of the whole T cell preparation with one or two magnetic antibody cell surface markers;

(b) removing the magnetically-tagged resistant T cell subset from the whole T cell preparation;

(c) staining the magnetically-depleted T cell preparation with a fluorescent membrane potential dye about 1 to about 250 nM per 2 x 10^6 T cells per mL;

(d) loading the fluorescent-stained, magnetically-depleted T cell preparation into an analytical instrument capable of measuring fluorescence;

(e) measuring a baseline membrane potential dye fluorescence of the fluorescent-stained, magnetically-depleted T cell preparation;

(f) adding the immunoregulant compound to the fluorescent-stained, magnetically-depleted T cell preparation;

(g) measuring the immunoregulant compound induced membrane potential dye fluorescence of the fluorescent-stained, magnetically-depleted T cell preparation containing the immunoregulant compound after about 30 seconds to about 5 minutes;

(h) adding anti-CD3 antibody or a biotinylated anti-CD3 to the fluorescent-stained, magnetically-depleted T cell preparation containing the immunoregulant compound;

(i) incubating the anti-CD3 antibody or a biotinylated anti-CD3 fluorescent-stained, magnetically-depleted T cell preparation containing the immunoregulant compound for about 3 to about 10 minutes;

(j) adding a crosslinker antibody or avidin to the anti-CD3 antibody or a biotinylated anti-CD3 labeled, fluorescent-stained, magnetically-depleted T cell preparation containing the immunoregulant compound;

(k) incubating the crosslinker antibody or avidin with the anti-CD3 antibody or a biotinylated anti-CD3 labeled, fluorescent-stained, magnetically-depleted T cell preparation containing the immunoregulant compound for about 2 minutes; and

(l) measuring the integrated anti-CD3 induced membrane potential dye fluorescence of the antibody crosslinked or avidin crosslinked anti-CD3 labeled, fluorescent-stained, magnetically-depleted T cell preparation containing the immunoregulant compound.

A third embodiment is the process for screening for immunoregulant compounds that modulate T cell activation of whole T cell preparations which comprises the steps of:

(a) loading whole T cell preparation with about 2 to about 10 μM total concentration of fluorescent calcium indicator dye and calcium chelator per 2 x 10^6 T cells per mL;

(b) staining the loaded T cell preparation with a fluorescent membrane potential dye about 1 to about 250 nM per 2 x 10^6 T cells per mL;

(c) placing the loaded and stained T cell preparation into an analytical instrument capable of measuring fluorescence;

(d) measuring a baseline membrane potential dye fluorescence of the loaded and stained T cell preparation;

(e) adding the immunoregulant compound to the loaded and stained T cell preparation;

(f) measuring the immunoregulant compound induced membrane potential dye fluorescence of the loaded and stained T cell preparation containing the immunoregulant compound after about 30 seconds to about 5 minutes;

(g) adding anti-CD3 antibody or a biotinylated anti-CD3 to the loaded and stained T cell preparation containing the immunoregulant compound;

(h) incubating the anti-CD3 antibody or the biotinylated anti-CD3 with the loaded and stained T cell preparation containing the immunoregulant compound for about 3 to about 10 minutes;

(i) adding a crosslinker antibody or avidin to the anti-CD3 antibody or a biotinylated anti-CD3 labeled, loaded and stained T cell preparation containing the immunoregulant compound;

(j) incubating the crosslinker antibody or avidin with the anti-CD3 antibody or a biotinylated anti-CD3 labeled, loaded and stained T cell preparation containing the immunoregulant compound for about 2 minutes; and

(k) measuring the integrated anti-CD3 induced membrane potential dye fluorescence of the antibody crosslinked or avidin

crosslinked anti-CD3 labeled, loaded and stained T cell preparation containing the immunoregulant compound.

All analytical instruments capable of measuring fluorescence are within the scope of this invention, and include but are not limited to for example a flow cytometer, digital imaging microscope, confocal microscope, spectrofluorimeter, including a confocal spectrofluorimeter. Additionally, the scope of this invention includes analytical instruments capable of measuring fluorescence which have a computer capable of analyzing only the fluorescence on antibody negative or susceptible cells, in cases where the T-cell subsets are unseparated. The analytical instrument capable of measuring fluorescence is configured to measure the fluorescence either continuously or at discrete time windows. Preferrably, the analytical instrument capable of measuring fluorescence is configured to measure the fluorescence at 1 to about 3 discrete time windows. The discrete time window is defined as a time period ranging from about 1 second to about 1 hour.

The membrane potential dyes , also referred to as potential-sensitive probes commonly used are largely fluorescent indicators comprising: styryl dyes, oxonols, carbocyanines and merocyanines. [<u>See</u> R. Haugland, "MOLECULAR PROBES, Handbook of Fluorescent Probes and Research Chemicals, Set 23: Potential-Sensitive Probes" (1992) pp 153-158, for a complete list of membrane potential dyes.] The preferred membrane potential dyes are: $DiBAC_4$, $DiSBAC_2$, and $DiOC_5$.

The membrane potential dye concentration used in the instant process is in the range of about 1 to about 250 nM per 2 x 10^6 T cells per mL for the cell subsets, and preferably, about 10 nM per 2 x 10^6 T cells per mL. The calcium chelating dye concentration used in the instant process for non-subset cells is in the range of about 1 to about 10 μM per 2 x 10^6 T cells per mL, and preferably, about 4 to about 6 μM per 2 x 10^6 T cells per mL. The critical consideration is the intracellular dye concentration which relates to the ratio of dye concentration to the total integrated cell surface area for the membrane potential dye and total integrated cell surface volume for the calcium chelator dye.

A preferred embodiment of this invention is the process which utilizes an analytical instrument capable of measuring fluorescence selected from: flow cytometer, digital imaging microscope, and confocal microscope; and the membrane potential dye is selected from the group consisting of: DiBAC4, DiSBAC2, and DiOC5.

Analytical Instruments Capable Of Measuring Fluorescence	Membrane potential/ antibody subsets	Membrane potential/ Magnetically separated subsets	Membrane potential/ Calcium Chelator
spectrofluorimeter	No	possible but not accurate	possible but not accurate
confocal spectrofluorimeter	No	DiBAC4 DiSBAC2 DiOC5	DiBAC4 DiSBAC2 DiOC5
flow cytometer	DiBAC4 DiSBAC2 DiOC5	DiBAC4 DiSBAC2 DiOC5	DiBAC4 DiSBAC2 DiOC5
Digital Imaging Microscope	DiBAC4 DiSBAC2 DiOC5	DiBAC4 DiSBAC2 DiOC5	DiBAC4 DiSBAC2 DiOC5
Confocal Microscope	DiBAC4 DiSBAC2 DiOC5	DiBAC4 DiSBAC2 DiOC5	DiBAC4 DiSBAC2 DiOC5

The invention relates to a method for screening compounds which are either competitive or allosteric modulators of peptide binding whose effect on early activation events, specifcally membrane potential and calcium transient is similar to the activity seen with margatoxin and is therefore useful in identifying compounds which suppress the immune system in a subject in need of such treatment comprising the

administration to a subject in need of such treatment of a nontoxic immunosuppressant amount of an inhibitor.

Compounds screened using this methodology are viewed to be active as immunoregulants when there is an increase in membrane potential dye fluorescence of about 1.5 to about 2 times above the background fluorescence after margatoxin inhibition, and about a 50% increase above the margatoxin depolarized level upon crosslinking. See Figure 1.

The high capacity screen of this invention utilizes cell surface markers on the susceptible T cell subsets with whole T cell preparations using fluorescent antibodies to cell surface markers and taking measurements on one of the following instruments: a multiparameter fluorescence flow cytometer, a multiparameter fluorescence confocal microscope or a multiparameter fluorescence digital imaging microscope.

The high capacity screen of this invention can also utilize magnetically separated susceptible T cell subsets to achieve reproducible results with human cells with measurements taken on one of the following instruments: a multiparameter fluorescence flow cytometer, a multiparameter fluorescence confocal microscope, a multiparameter fluorescence digital imaging microscope, a confocal spectrofluorimeter or a spectrofluorimeter. This reproducibility is not possible on whole T cells because of individual variations in the percent of T cells which are unresponsive to $K_V1.3$ blockers.

The high capacity screen of this invention can also utilize unseparated T cells loaded with a high concentration calcium chelator dye which renders all T cells susceptible to $K_V1.3$ blockers. This allows for screening a whole (unseparated) T cell preparation in a reproducible manner.

Specifically, the method of this invention is useful in screening for compounds that possess $K_V1.3$ channel inhibitory activity. $K_V1.3$ channel inhibitors are useful in the treatment and prevention of the resistance to transplantation or transplantation rejection of organs or tissues (such as heart, kidney, liver, lung, bone marrow, cornea, pancreas,

intestinum tenue, limb, muscle, nervus, medulla ossium, duodenum, small-bowel, medulla ossium, skin, pancreatic islet-cell, etc. including xeno transplantation), graft-versus-host diseases by medulla ossium transplantation, autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematosis, nephrotic syndrome lupus, Hashimoto's thyroiditis, multiple sclerosis, myasthenia gravis, type I diabetes mellitus, type II adult onset diabetes, uveitis, nephrotic syndrome, steroid-dependent and steroid-resistant nephrosis, Palmo-planter pustulosis, allergic encephalomyelitis, glomerulonephritis, etc., and infectious diseases caused by pathogenic microorganisms, as well as being useful to screen for compounds useful in treating inflammatory, proliferative and hyperproliferative skin diseases and cutaneous manifestations of immunologically-mediated illnesses such as: psoriasis, psoriatic arthritis, atopical dermatitis, contact dermatitis and further eczematous dermatitises, seborrhoeic dermatitis, Lichen planus, Pemphigus, bullous Pemphigoid, Epidermolysis bullosa, urticaria, angioedemas, vasculitides, erythemas, cutaneous eosinophilias, acne, Alopecia areata, eosinophilic fasciitis, and atherosclerosis.

$K_V1.3$ channel inhibitors are also useful in the treatment of respiratory diseases, for example: sarcoidosis, fibroid lung, idiopathic interstitial pneumonia, and reversible obstructive airways disease, including conditions such as asthma, including bronchial asthma, allergic asthma, intrinsic asthma, extrinsic asthma and dust asthma, particularly chronic or inveterate asthma (for example late asthma and airway hyper-reponsiveness), bronchitis and the like. $K_V1.3$ channel inhibitors may also be useful for treating hepatic injury associated with ischemia.

$K_V1.3$ channel inhibitors may also useful in the treatment of certain eye diseases such as keratoconjunctivitis, vernal conjunctivitis, uveitis associated with Behcet's disease, keratitis, herpetic keratitis, conical cornea, dystorphia epithelialis corneae, corneal leukoma, ocular pemphigus, Mooren's ulcer, Scleritis, Graves' ophthalmopathy, severe intraocular inflammation, and the like. A $K_V1.3$ channel blocker is also useful for treating multidrug resistance of tumor cells, (i.e. enhancing the activity and/or sensitivity of chemotherapeutic agents), preventing or

treating inflammation of mucosa or blood vessels (such as leukotriene B4-mediated diseases, gastric ulcers, vascular damage caused by ischemic diseases and thrombosis, ischemic bowel disease, inflammatory bowel disease (e.g., Crohn's disease and ulcerative colitis), necrotizing enterocolitis), or intestinal lesions associated with thermal burns, cytomegalovirus infection, particularly HCMV infection.

Further, $K_V1.3$ channel blockers are also useful for treating or preventing renal diseases including interstitial nephritis, Goodpasture's syndrome, hemolytic-uremic syndrome and diabetic nephropathy; nervous diseases selected from multiple myositis, Guillain-Barre syndrome, Meniere's disease and radiculopathy; endocrine diseases including hyperthyroidism and Basedow's disease; hematic diseases including pure red cell aplasia, aplastic anemia, hypoplastic anemia, idiopathic thrombocytopenic purpura, autoimmune hemolytic anemia, agranulocytosis and anerythroplasia; bone diseases including osteoporosis; respiratory diseases including sarcoidosis, fibroid lung and idiopathic interstitial pneumonia; skin diseases including dermatomyositis, leukoderma vulgaris, ichthyosis vulgaris, photoallergic sensitivity and cutaneous T cell lymphoma; circulatory diseases including arteriosclerosis, aortitis syndrome, polyarteritis nodosa and myocardosis; collagen including scleroderma, Wegener's granuloma and Sjogren's syndrome; adiposis; eosinophilic fasciitis; periodontal disease; nephrotic syndrome; hemolytic-uremic syndrome; and muscular dystrophy. Further still, $K_V1.3$ channel blockers, may be used in the treatment of diseases including intestinal inflammations/allergies such as Coeliac disease, proctitis, eosinophilic gastroenteritis, mastocytosis, Crohn's disease and ulcerative colitis; and food-related allergic diseases which have symptomatic manifestation remote from the gastrointestinal tract, for example migraine, rhinitis and eczema.

$K_V1.3$ channel blockers may also be useful for the treatment and prevention of hepatic diseases such as immunogenic diseases (e.g. chronic autoimmune liver diseases including autoimmune hepatitis, primary biliary cirrhosis and sclerosing cholangitis), partial liver resection, acute liver necrosis (e.g. necrosis caused by toxins, viral

hepatitis, shock or anoxia), B-virus hepatitis, non-A/non-B hepatitis and cirrhosis.

The following example is given for the purpose of illustrating the present invention and shall not be construed as being limitations on the scope or spirit of the invention.

EXAMPLE 1

Materials and Methods

Reagents: MgTX was purified from the venom of the scorpion *Centruroides margaritatus* as described by M. Garcia-Calvo. See Garcia-Calvo M., R.J. Leonard, J. Novick, S.P Stevens., W. Schmalhofer, G.J Kaczorowski., and M.L. Garcia. "Purification, characterization, and biosynthesis of margatoxin, a component of Centruoides margaritatus venom that selectively inhibits voltage-dependant potassium channels" . J Biol Chem 268.5: 18866-18874 (1993). All antibody preparations (biotin-anti-CD3, PE-anti-CD4 and PE-anti-CD8) were obtained from Becton Dickinson. The biotin-anti-CD3 was diluted 1:10 with media for use. Avidin (#A9890 Sigma Chemical Company, Saint Louis, MO) was dissolved in phenol-red-free RPMI 1640 medium (GIBCO, Grand Island NY) at 1 mg/ml, filter sterilized and maintained at 4° C until diluted 1:5 with medium for use. A 1 mM stock solution DiBAC4(3) (Molecular Probes, Eugene, OR) was made in EtOH and maintained in the dark at room temperature until use. [See Wilson H.A., and T.M. Chused. 1985. Lymphocyte membrane potential and Ca2+ sensitive potassium channels described by Oxinol dye fluorescence measurements . J Cell Physiol 125: 72-81; and Wilson H.A., B.E. Seligmann, and T.M. Chused. 1985. Voltage Sensitive Cyanine Dye Fluorescence signals in Lymphocytes: plasma membrane and mitochondrial components. J Cell Physiol 125: 61-71.]. A 1 mM stock solution $DiSBAC_2(3)$ (Molecular Probes, Eugene, OR) was made in EtOH and maintained in the dark at room temperature until use.

Cell Preparations: Purified T cells were prepared by a modified "E-rosetting " method [Lin C.S., R.C. Boltz., J.J. Siekierka, and N.H. Sigal. "FK-506 and Cyclosporin A inhibit highly similar signal transduction pathways in human T lymphocytes." Cellular Immunology 133: 269 (1991).] from lymphocyte-rich leucopaks. The purified cells were washed in lymphocyte cell culture medium, RPMI 1640 medium (GIBCO, Grand Island NY) and maintained in serum supplemented RPMI at 37° C for use within 24 hrs. The cells were washed in HEPES-buffered, phenol-red-free RPMI prior to staining. All subsequent fluorescent procedures were performed in this media. The T cells were separated into CD4 enriched ($CD8^-$) and CD8 enriched ($CD4^-$) populations using a MACS magnetic cell separator (Miltenyi Biotec GmbH, Bergish-Gladbach, Germany) according to product instructions.

Measurement of Membrane Potential.

Flow Cytometry: T-cells were washed and re-suspended at 5×10^6 cells/ml. The cells were maintained in the dark until use or staining. When cells were stained for subset discrimination, either anti-CD4 or anti-CD8 was added to 200 µL of 5×10^6 cells/ml, washed and re-suspended at 1×10^7 cells/ml. A stock solution of 1 mM DiBAC4(3), a membrane potential dye, was diluted 1:200 in double-distilled H_2O and then further diluted 1:99 in phenol red free RPMI. The solution was mixed with the cell suspension at 1:9 and equilibrated 15 minutes at room temperature. Flow cytometric time course determinations were performed as previously described in Lin C.S., et al. Cellular Immunology 133: 269 (1991) . Multiparameter studies were carried out at 37° C on a FACStar Plus/Consort VAX (Becton Dickinson Immunocytometry Systems, San Jose, CA) equipped with a Zero Time Module (CYTEK Corp. Freemont, CA). Additions for of the inhibitor or mock inhibitor (media) were at 30 seconds, biotinylated anti-CD3 at 200 seconds, and avidin at 400 seconds. Cell data was accumulated as a single continuous, 8 parameter list-mode file with 10 time indicators/sec. Mean time course plots were calculated from the list mode data using KINPRO (Becton Dickinson Immunocytometry Systems, San Jose, CA).

Digital Imaging Microscopy: For example, the digital imaging microscope components were purchased through Perceptics Corporation (Knoxville, TN). The components of system used in this study were an inverted Axiovert 35 microscope (Zeiss, Thornwood NY) fitted with a frame-transfer cooled-CCD camera (CH220 with a TC312 1024 x 512 masked chip, Photometrics, Tucson AZ). Excitation filters, 340 DF 10 and 380 DF 13 (Omega Optical,Inc., Brattleboro VT), were controlled via a control device through an RS 232 connection (Ludl Electronic Products, Hawthorne, NY). System control, image recording and analysis was through TCL Image software (TNO, Delft Netherlands) operating on Apple Macintosh IIx. Command macros were written and tested in-house for acquisition and analysis of image pairs.

Two adjacent chambers of each Lab-Tek 8 chambered cover-glass (NUNC Inc., Naperville IL) were coated with 0.8μg/cm^2 Cell-Tak (Collaborative Research In. Bedford MA) following the product usage instructions. The chambers were stored at 4° C until the day of the experiment. The chambers were equilibrated at room temperature prior to plating of the cells.

The T-cells were loaded with 5 nM DiOC5(3) cyanine dye (Molecular Probes) following the same procedure used above for DiBAC4 but were re-suspended at a concentration of 1 x 10^6/ml. The cells were maintained in the dark until plating. At least 2.5 hrs prior to time course acquisition, 150μL of the suspension was plated in the Cell-Tak coated chambers. Ten minutes prior to the data acquisition, T-cells were loaded with 5 nM DiOC5(3) cyanine dye (Molecular Probes) the chamber was placed in the heating stage and the addition pipets filled and placed in the heating block. Both were warmed to 37° C.

An external heating unit maintains the temperature of the cells in the disposable NUNC chamber as previously described in Boltz, R.C.D., G. Kath, B. Uhrig, J. McKeel, and C. Quinn. "A disposable-chamber temperature-regulation system for the study of intracellular calcium levels in single live T cells using fluorescence digital-imaging microscopy'. Accepted for publication Cytometry 17:2 October, 1994.

Pre-warming of addition solutions was accomplished in a separately regulated addition block. Prior to the initiation of the membrane potential time course, disposable Eppendorf tips were filled with the appropriate solutions and placed in the addition block for pre-heating to 37^{o} C. During the experiment, additions were made by gently pushing the remote syringe plunger. By using addition volumes equal to that of the cell suspension, complete mixing was achieved.

In order to measure changes in membrane potential, single images were accumulated and stored every 10 seconds. Computer clock time was stored with each image. Each time course was stored on a REO-650 erasable optical disc (Pinnacle Micro, Irvine CA). Briefly, for analysis, cells were defined and numbered with a fluorescence threshold mask. Fluorescence intensity for each time point was calculated. The area normalized integrated intensity of each cell was placed in an EXCEL type spreadsheet in the appropriate cell column in a row designated with the experiment time calculated from the computer clock time. The individual time course of the single cells was displayed using StatView II (Abacus Concepts, Berkeley CA).

WHAT IS CLAIMED IS:

1. A process for screening for immunoregulant compounds that modulate T cell activation which comprises measuring the effect of the immunoregulant compound on membrane potential.

2. A process for screening for immunoregulant compounds that modulate T cell activation of whole T cell preparations which comprises the steps of:

(a) tagging a resistant T cell subset of the whole T cell preparation with one or two fluorescent antibody cell surface markers;

(b) staining the tagged whole T cell preparation with a fluorescent membrane potential dye about 1 to about 250 nM per 2 x 10^6 T cells per mL;

(c) loading the fluorescent-stained, tagged whole T cell preparation into an analytical instrument capable of measuring fluorescence;

(d) measuring a baseline membrane potential dye fluorescence of the fluorescent-stained, tagged whole T cell preparation;

(e) adding the immunoregulant compound to the fluorescent-stained, tagged whole T cell preparation;

(f) measuring the immunoregulant compound induced membrane potential dye fluorescence of the fluorescent-stained, tagged whole T cell preparation containing the immunoregulant compound after about 30 seconds to about 5 minutes;

(g) adding anti-CD3 antibody or a biotinylated anti-CD3 to the fluorescent-stained, tagged whole T cell preparation containing the immunoregulant compound;

(h) incubating the anti-CD3 antibody or a biotinylated antiCD-3, fluorescent-stained, tagged whole T cell preparation containing the immunoregulant compound for about 3 to about 10 minutes;

(i) adding a crosslinker antibody or avidin to the anti-CD3 antibody or a biotinylated anti-CD3 labeled, fluorescent-

stained, tagged whole T cell preparation containing the immunoregulant compound;

(j) incubating the crosslinker antibody or avidin the anti-CD3 antibody or a biotinylated anti-CD3 labeled, fluorescent-stained, tagged whole T cell preparation containing the immunoregulant compound for about 2 minutes: and

(k) measuring the integrated anti-CD3 induced membrane potential dye fluorescence of the antibody crosslinked or avidin crosslinked anti-CD3 labeled, fluorescent-stained, tagged whole T cell preparation containing the immunoregulant compound.

3. The process for screening for immunoregulant compounds that modulate T cell activation of whole T cell preparations, as recited in claim 2, wherein the analytical instrument capable of measuring fluorescence comprises: a flow cytometer, digital imaging microscope or confocal microscope.

4. The process for screening for immunoregulant compounds that modulate T cell activation of whole T cell preparations, as recited in claim 3, wherein the analytical instrument capable of measuring fluorescence further comprises a computer capable of analyzing only the fluorescence on antibody negative or susceptible cells.

5. The process for screening for immunoregulant compounds that modulate T cell activation of whole T cell preparations, as recited in claim 3, wherein the analytical instrument capable of measuring fluorescence is configured to measure the integrated fluorescence continuously or at discrete time windows.

6. The process for screening for immunoregulant compounds that modulate T cell activation of whole T cell preparations, as recited in claim 5, wherein the analytical instrument capable of

measuring fluorescence is configured to measure the integrated fluorescence at 1 to about 3 discrete time windows.

7. The process for screening for immunoregulant compounds that modulate T cell activation of whole T cell preparations, as recited in claim 3, wherein the fluorescent membrane potential dye comprises: styryl dyes, oxonols, carbocyanines or merocyanines.

8. The process for screening for immunoregulant compounds that modulate T cell activation of whole T cell preparations, as recited in claim 7, wherein the analytical instrument capable of measuring fluorescence comprises: flow cytometer, digital imaging microscope, and confocal microscope; and the fluorescent membrane potential dye comprises: $DiBAC_4$, $DiSBAC_2$, or $DiOC_5$.

9. The process for screening for an immunoregulant compound that modulates T cell activation of whole T cell preparations, as recited in claim 2, wherein the concentration of fluorescent membrane potential dye is about 10 nM per 2×10^6 T cells per mL.

10. A process for screening for immunoregulant compounds that modulate T cell activation of whole T cell preparations which comprises the steps of:

(a) tagging a resistant T cell subset of the whole T cell preparation with one or two magnetic antibody-cell surface markers;

(b) removing the magnetically-tagged resistant T cell subset from the whole T cell preparation;

(c) staining the magnetically-depleted T cell preparation with a fluorescent membrane potential dye about 1 to about 250 nM per 2×10^6 T cells per mL;

(d) loading the fluorescent-stained, magnetically-depleted T cell preparation into an analytical instrument capable of measuring fluorescence;

(e) measuring a baseline membrane potential dye fluorescence of the fluorescent-stained, magnetically-depleted T cell preparation;

(f) adding the immunoregulant compound to the fluorescent-stained, magnetically-depleted T cell preparation;

(g) measuring the immunoregulant compound induced membrane potential dye fluorescence of the fluorescent-stained, magnetically-depleted T cell preparation containing the immunoregulant compound after about 30 seconds to about 5 minutes;

(h) adding anti-CD3 antibody or a biotinylated anti-CD3 to the fluorescent-stained, magnetically-depleted T cell preparation containing the immunoregulant compound;

(i) incubating the anti-CD3 antibody or a biotinylated anti-CD3 fluorescent-stained, magnetically-depleted T cell preparation containing the immunoregulant compound for about 3 to about 10 minutes;

(j) adding a crosslinker antibody or avidin to the anti-CD3 antibody or a biotinylated anti-CD3 labeled, fluorescent-stained, magnetically-depleted T cell preparation containing the immunoregulant compound;

(k) incubating the crosslinker antibody or avidin with the anti-CD3 antibody or a biotinylated anti-CD3 labeled, fluorescent-stained, magnetically-depleted T cell preparation containing the immunoregulant compound for about 2 minutes; and

(l) measuring the integrated anti-CD3 induced membrane potential dye fluorescence of the antibody crosslinked or avidin crosslinked anti-CD3 labeled, fluorescent-stained, magnetically-depleted T cell preparation containing the immunoregulant compound.

11. A process for screening for immunoregulant compounds that modulate T cell activation of whole T cell preparations which comprises the steps of:

(a) loading whole T cell preparation with about 2 to about 10 μM total concentration of fluorescent calcium indicator dye and calcium chelator per 2 x 10^6 T cells per mL;

(b) staining the loaded T cell preparation with a fluorescent membrane potential dye about 1 to about 250 nM per 2 x 10^6 T cells per mL;

(c) placing the loaded and stained T cell preparation into an analytical instrument capable of measuring fluorescence;

(d) measuring a baseline membrane potential dye fluorescence of the loaded and stained T cell preparation;

(e) adding the immunoregulant compound to the loaded and stained T cell preparation;

(f) measuring the immunoregulant compound induced membrane potential dye fluorescence of the loaded and stained T cell preparation containing the immunoregulant compound after about 30 seconds to about 5 minutes;

(g) adding anti-CD3antibody or a biotinylated anti-CD3 to the loaded and stained T cell preparation containing the immunoregulant compound;

(h) incubating the anti-CD3 antibody or the biotinylated anti-CD3 with the loaded and stained T cell preparation containing the immunoregulant compound for about 3 to about 10 minutes;

(i) adding a crosslinker antibody or avidin to the anti-CD3 antibody or a biotinylated anti-CD3 labeled, loaded and stained T cell preparation containing the immunoregulant compound;

(j) incubating the crosslinker antibody or avidin with the anti-CD3 antibody or a biotinylated anti-CD3 labeled, loaded and stained T cell preparation containing the immunoregulant compound for about 2 minutes; and

(k) measuring the integrated anti-CD3 induced membrane potential dye fluorescence of the antibody crosslinked or avidin crosslinked anti-CD3 labeled, loaded and stained T cell preparation containing the immunoregulant compound.

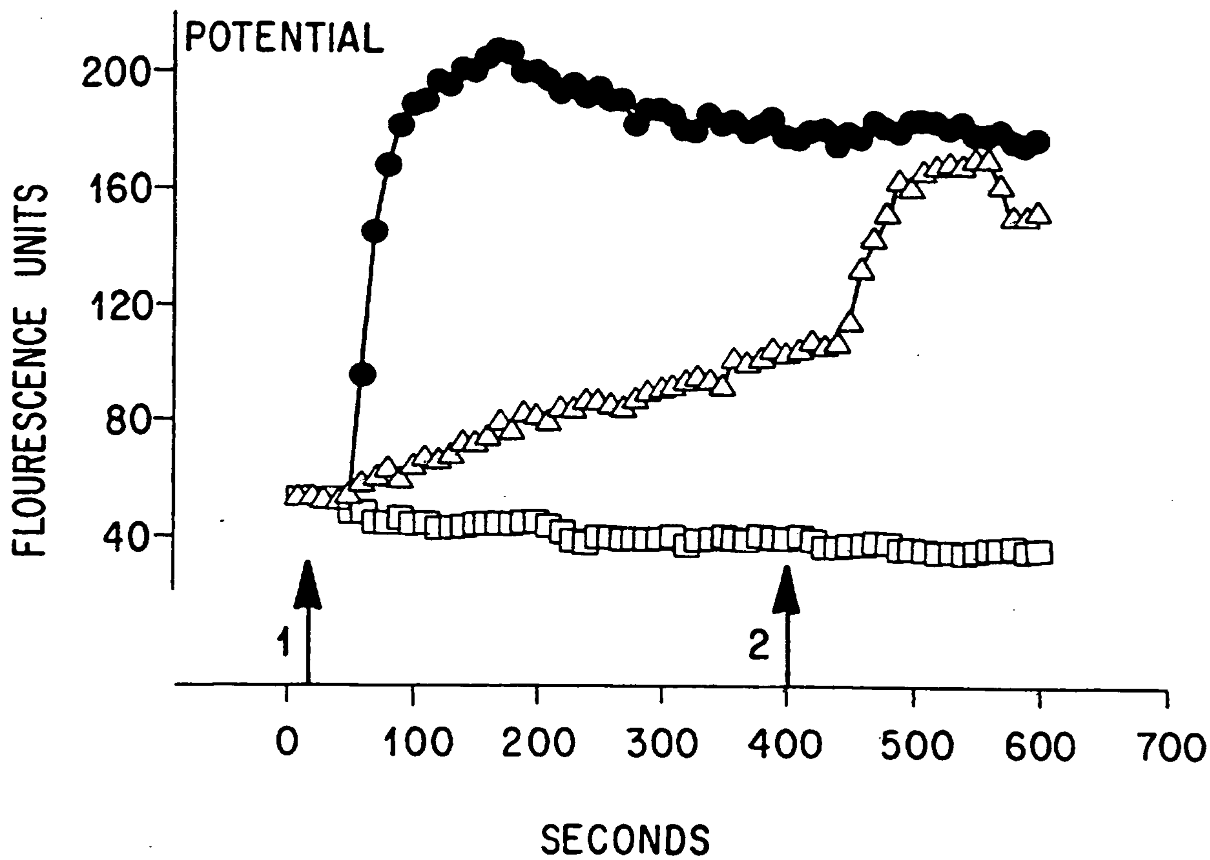
POTENTIAL
200
160
120
80
40
FLOURESCENCE UNITS
1
2
0
100
200
300
400
500
600
700
SECONDS

FIG. 1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/12316

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12Q 1/02, 1/24
US CL :435/7.24, 29, 30
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.24, 29, 30

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS: T(W)(CELL# OR LYMPHOCYTE#) AND MEMBRANE(W)POTENTIAL

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ---- A	Proceedings of the National Academy of Sciences USA, Volume 89, issued November 1992, R.J. LEONARD ET AL, "Selective blockers of voltage-gated K^+ channels depolarize human T lymphocytes: Mechanism of the antiproliferative effect of charybdotoxin", pages 10094-10098. See page 10097, column 2, first full paragraph; page 10098, column 1, second paragraph.	1 ------- 2-11
X ---- A	WO, A, 90/10871 (THE REGENTS OF THE UNIVERSIY OF CALIFORNIA) 20 September 1990. See page 7, lines 4-15; page 11, lines 4-12.	1 ------ 2-11
X, P ------- A, P	US, A, 5,397,702 (CAHALAN ET AL) 14 March 1995. See column 14, lines 21-28.	1 ------ 2-11

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
14 NOVEMBER 1995	27 NOV 1995

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